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Production of "biobetter" glucarpidase variants to improve Drug Detoxification and Antibody Directed Enzyme Prodrug Therapy for Cancer Treatment

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Abstract

Recombinant glucarpidase (formerly: Carboxypeptidase G2, CPG2) is used in Antibody Directed Enzyme Prodrug Therapy (ADEPT) for the treatment of cancer. In common with many protein therapeutics, glucarpidase has a relatively short half-life in serum and, due to the need for the repeated cycles of the ADEPT, its bioavailability may be further diminished by neutralizing antibodies produced by patients. PEGylation and fusion with human serum albumin (HSA) are two approaches that are commonly employed to increase the residency time of protein therapeutics in blood, and also to increase the half-lives of the proteins in vivo. To address this stability and the immunogenicity problems, 'biobetter' glucarpidase variants, mono-PEGylated glucarpidase, and HSA fused glucarpidase by genetic fusion with albumin, were produced. Biochemical and bioactivity analyses, including anti-proliferation, bioassays, circular dichroism, and in vitro stability using human blood serum and immunoassays, demonstrated that the functional activities of the designed glucarpidase conjugates were maintained. The immunotoxicity studies indicated that the PEGylated glucarpidase did not significantly induce T-cell proliferation, suggesting that glucarpidase epitopes were masked by the PEG moiety. However, free glucarpidase and HSA-glucarpidase significantly increased T-cell proliferation compared with the negative control. In the latter case, this might be due to the type of expression system used or due to trace impurities associated with the highly purified (99.99%) recombinant HSA-glucarpidase. Both PEGylated glucarpidase and HAS-glucarpidase exhibit more stability in human serum and were more resistant to key human proteases relative to native glucarpidase. To our knowledge, this study is the first to report stable and less immunogenic glucarpidase variants produced by PEGylation and fusion with HSA. The results suggest that they may have better efficacy in drug detoxification and ADEPT, thereby improving this cancer treatment strategy.

Keywords

Carboxypeptidase G2; CPG2; Antibody Directed Enzyme Prodrug Therapy; ADEPT; PEGylation; human serum albumin; HSA; HSA-glucarpidase; PEGylated glucarpidase; Cancer.

Introduction

The US Food and Drug Administration has approved more than 180 therapeutic peptides and proteins for many applications and disease treatments. Because most of these proteins and peptides are smaller in size than the kidney filtration cutoff of ~70 kDa, they do not have optimal pharmacokinetics. Thus, these protein and peptide therapeutics have short half-lives *in vivo*, due to the action of proteases and the generation of antibodies against them [1].

Due to these features, therapeutic proteins and peptides needed to be injected frequently and in high dose. This not only results in the need for more frequent treatments, often leading to patient non-compliance, but it also results in reduced drug efficacy.

One of the therapeutic proteins used in cancer therapy is glucarpidase, also known as Carboxypeptidase G2, CPG2, which originates from the bacterium *Variovorax paradoxus* (old name, *Pseudomonas* sp. strain RS-16). It has no mammalian equivalent [2, 3] and is a zinc-dependent dimeric protein with two subunits of 41 kDa [4, 5]. Glucarpidase has a relatively restricted specificity and hydrolyzes the C-terminal glutamic acid residue of folic acid and folate analogues such as methotrexate [6]. The mechanism of action of glucarpidase is, therefore, to lower systemic methotrexate levels by rapidly causing methotrexate to be converted to glutamate and 4-deoxy-4-amino-N 10-methylpteroic acid (DAMPA), both of which undergo hepatic metabolism.

Glucarpidase, consequently, provides an alternative route for methotrexate elimination in patients with impaired renal function. This action of glucarpidase on methotrexate makes the enzyme not only a powerful rescue agent in patients receiving high doses of methotrexate but also helps to avoid life-threatening toxicity in patients with methotrexate intoxications.

The enzyme can also be used in a targeted cancer therapy technique known as Antibody Directed Enzyme Prodrug Therapy (ADEPT), which has already been implemented for cancer treatment [7].

ADEPT consists of two steps (fig. 1), which result in the production of a powerful cytotoxic drug only in the vicinity of the tumor. In the first step, a tumor-selective antibody is chemically linked to an enzyme and then administered intravenously to the patient. The second step includes the injection of a non-toxic drug precursor (Prodrug).

The enzyme, which accumulates at the tumor site via the tumor-specific antibody, converts the prodrug into an active drug. This therapy, therefore, produces a powerful cytotoxic drug in the vicinity of the tumor with little toxicity elsewhere in the patient body. One of the enzymes that has been used in the

ADEPT is the glucarpidase from *V. paradoxus* strain RS-16, which when applied has been shown to result in antitumor activity in different types of cancer [8-11].

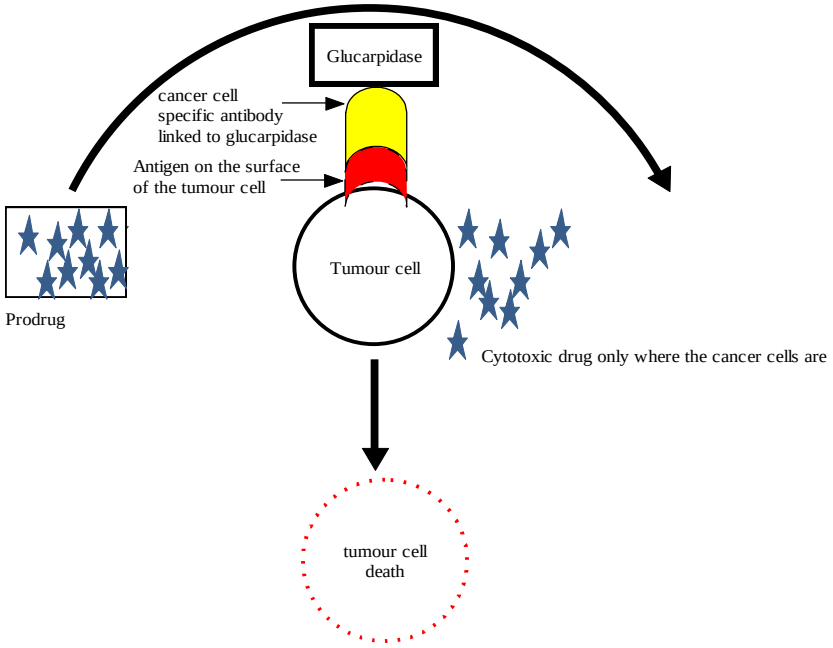


Fig. 1. The principle of ADEPT. The first step is the injection of the antibody-enzyme fusion protein, which localizes to tumors. The second step is the injection of a prodrug, following clearance of the fusion protein from the blood. The localized glucarpidase converts the prodrug into the cytotoxic drug, which can diffuse through the tumor and kill antigen-containing tumor cells as well as tumor cells that are nearby but do not express the relevant antigen, thus giving a "bystander" effect [12].

The use of glucarpidase in the ADEPT and the drug detoxification are effective but have limitations. The treatment requires repeated cycles, which results in a severe immune response against the glucarpidase and, additionally, proteases present in patients' blood degrade the enzyme thereby limiting its therapeutic applicability.

We recently isolated a new glucarpidase that shares 94% amino acid identity with the one produced by *V. paradoxus* strain RS-16 [13]. We also demonstrated that antibodies raised against the newly isolated glucarpidase do not react with the one from *V. paradoxus* strain RS-16.

In this work, we report the production of long-acting variants of our glucarpidase to overcome the complications related to the multiple cycles of ADEPT. A number of strategies have been developed to address the issue of immunogenicity and to improve the pharmacokinetics of protein and peptide therapeutics. These include PEGylation, i.e. the attachment of polyethylene glycol (PEG) polymer chains,

fusion with human serum albumin (HSA), fusion with non-structured polypeptides, and fusion with the constant fragment (Fc) domain of a human immunoglobulin (Ig)G [14]. In this study, we focused our work on the production of 'biobetter' glucarpidases by using PEGylation (PEG) and fusion with the Human serum albumin (HSA).

PEGylation technology has already been used successfully to produce long-acting proteins. For example, PEGylated forms of interferon α2b and interferon α2a, which are known commercially as Pegintron and Pegasys respectively, have been used for the treatment of treatment of patients with melanoma and hepatitis B. Similarly, a PEGylated version of granulocyte colony-stimulating factor has been used for the treatment of chemotherapy-induced neutropenia [15-17].

Human serum albumin, which has a circulation half-life of nineteen days[18], has also been used to extend the half-life of biopharmaceuticals and to maintain their bioactivity[19, 20]. Protein therapeutics that have been improved using this strategy include vascular endothelial growth factor [21], interferon [22, 23], and interleukin-2 [24].

In this study, we implemented the two strategies, PEGylation of Lys residues and genetic fusion with human serum albumin, to produce long-acting glucarpidases to overcome current problems with ADEPT in cancer treatment.
Glucarpidase is clinically important enzyme in the antibody directed enzyme prodrug therapy and also in drug detoxification. Both applications have several pitfalls. We report, for first time, the production of novel glucarpidase variants with long acting and higher stability features than the free enzyme. Our work will pave the way for clinical investigation and in vivo studies.

Experimental Section

Bacterial strains, plasmids, and growth conditions.

The following strains of *E. coli*: Mach1™ T1R cells, DH5α™ *E. coli*, both from Thermo Fischer Scientific were used as cloning hosts and for plasmid propagation. Other strains such as BL21(DE3) RIL, Bl21 (DE3) and Rosetta™(DE3) Competent Cells (Novagen) were used for protein expression of the recombinant proteins. The plasmid pEX-K4 (Eurofins) was used for subcloning of the CPG2 to be conjugated with HSA, and pET28a (Novagen, Stratagen) was used for recombinant protein expression in *E. coli*. All recombinant bacterial strains were grown in Luria Bertani broth (Sigma life sciences) for liquid culture, which was solidified with agar (Sigma life sciences) for solid culture media.

Restriction enzymes, Antibodies and other chemicals

Restriction endonucleases and other enzymes required for cloning were from New England Biolabs. *Nde*I/*Hin*dIII were used for subcloning of CPG2 into pEX-K4-HSA, and *Kpn*I/ *Eco*R1 and *Hin*dIII were used to release the new conjugate into pET28a for expression study, and Calf Intestinal Alkaline phosphatase was used for vector dephosphorylation. T4 DNA ligase (Thermo Fischer Scientific) was used for the ligation step. T7 promoter: 5´-TAATACGACTCACTATAGGG-3´ and T7 terminator 5´-GCTAGTTATTGCTCAGCGG-3´ primers were obtained from Eurofins and used as universal primers for sequencing and subcloning confirmation. For protein purification by nickel affinity chromatography, Ni-NTA resin (Sigma) was used. Quick-Load® Purple 1 kb DNA Ladder (NEB) was used as the DNA marker while the SeeBlue Plus2 Prestained ladder (198-10 kDa) (Thermo Fischer Scientific) was used as protein markers. The GeneJET Plasmid Miniprep and Gel Extraction Kits (Thermo Fischer Scientific) were used for plasmid mini preparations and for DNA extraction and purification from gels, respectively. NuPAGE 10% Bis-Tris Gel Novex (Life Technologies) was used for SDS-PAGE Nitrocellulose Membrane (Thermo Fischer Scientific) was used for immunoblotting. Dialysis tube (Spectra/Por 7 Dialysis Tubing, 10 kDa MWCO, 24 mm Flat-width, 5 meters/roll (16 ft) (Spectrum) and dialysis tube of 50 kDa MWCO (Fischer Scientific) were used for exchanging buffers for proteins. The PEGylation reagent used was Y-shape (MPEG20K) 2-Succinimidyl Carboxymethyl Ester, MW 40000 (JenKem Technology). The following antibodies were used: mouse 6x-His Tag Monoclonal Antibody (HIS.H8) (Invitrogen) (1:2000 dilution), rabbit polyclonal anti Xen-CPG2 antibody (GE Healthcare) (1:2000 dilution), mouse anti-human serum albumin antibody [15C7] (ab10241) (Abcam) (1:500 dilution), rabbit anti-polyethylene glycol antibody [PEG-B-47] (ab51257) Abcam (1:2000 dilution) were used as primary antibodies for protein detection and goat anti-Mouse IgG H&L (HRP) (ab205719) from Abcam (1:4000 dilution) and goat anti-Rabbit IgG H&L (HRP) (ab6721) from Abcam (1:5000 dilution) were used as secondary conjugated antibodies. An ECL chemiluminescent detection reagent (GE Healthcare) was used as the substrate for detection of bound antibodies in western blotting.

Designing and construction Human Serum Albumin (HSA) to Xen-CPG2

The Human Serum Albumin (HSA) gene was custom synthesized by Eurofins genomics and inserted into the vector pEX-K4. The resulting construct was transformed into competent *E. coli* DH5alpha for propagation. pEX-K4-HSA was digested using *Nde*I/ *Hin*dIII and used as the receiving vector for insertion of a similarly digested DNA fragment carrying Xen-CPG2, which originated from a pET28a vector. Plasmid DNA from the resulting construct, designated pEX-K4-HSA-CPG2 and carrying a gene coding for an HSA-CPG fusion, was digested with *Kpn*I/*Hin*dIII to release the HSA-CPG2 gene fusion and then further digested with *Eco*RI prior to insertion into the *Eco*RI/*Hin*dIII digested expression vector

pET28a. The structure of the resulting construct, pET28a-Xen CPG2-HSA, was checked by restriction digestion and sequencing using T7 promoter and terminator universal primers.

Protein Expression of HSA Xen-CPG2 protein

E. coli BL21(DE3)RIL cells containing the pET28a-Xen CPG2-HSA were grown in LB medium supplemented with kanamycin and chloramphenicol at final concentrations of 33 µg/mL and 34 µg/mL, respectively. Following overnight incubation at 37 °C with shaking (200 rpm), 5 ml of the culture was added to 1L of fresh LB-broth containing the required antibiotics. The culture was incubated at 37 °C for 4 hours in an incubator shaker until the optical density at 600 nm was 0.5-0.6, at which point the culture was induced using isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM or left uninduced as the control. Following further incubation for four hours at 37 °C at 200 rpm, cells were harvested by centrifugation for 15 min at 4 °C and 4000 rpm. Cell pellets were re-suspended in 20 mM Tris buffer, pH 8 and 50 mM NaCl and were disrupted by sonication (MSE Soniprep 150 Plus) on ice (8 cycles of 30 sec pulses followed by 30 sec rest). Cell lysates were centrifuged at 14,000 rpm for 30 min at 4 °C for separation of the soluble and insoluble fractions. Each fraction was mixed with an equal volume of 2X sample buffer, denatured by boiling for 10 minutes at 95 °C, and then analyzed by SDS-PAGE. For maximum soluble protein production, cultures were induced with IPTG overnight at 20 °C.

Ni-NTA Purification of the HSA Xen-CPG2

The Xen-HSA CPG2 fusion protein was designed with a six-histidine tag at its N-terminus. Consequently, this protein was purified by Ni-NTA affinity chromatography. The resin was washed with sterile Milli-Q water and activated using a binding-washing buffer containing Tris (20 mM) pH 8, NaCl (50 mM), BME (5 mM), and imidazole (20 mM). Total soluble protein from *E. coli* BL21(DE3)RIL cells containing the pET28a-Xen CPG2-HSA was incubated with the resin with gentle agitation for 30 min at 4 °C. The combined resin was separated by gravity, the flow-through collected, and then the resin was washed repeatedly with wash buffer. The target protein bound to the resin was released by adding ice-cold elution Tris buffer containing 400 mM imidazole. The eluted protein was dialyzed against 100 mM Tris-HCl pH 7.3 containing 0.2 mM $ZnSO_4$ for activity assay. All collected fractions of protein purification steps were analyzed by SDS-PAGE.

Conjugation of Poly Ethylene Glycol (PEG) into Xen-CPG2

The purified CPG2, at a concentration of 0.5mg/ml, was dialyzed against 1X PBS pH 6 and subjected PEGylation by addition 1–5 fold molar excess of PEGylation reagent Y-shape (MPEG20K)2 Succinimidyl Carboxymethyl (SCM) Ester of MW 40,000 (JenKem Technology) in 100 mM sodium phosphate at pH 6, at room temperature for 30 min – 4 h. The same reaction was carried out at 30 °C, The PEGylation reaction was followed by SDS Gel analysis and the incubation time was prolonged to optimize the PEG binding to the CPG2 protein using a modification of the previous method[25].

. Larger scale PEGylation was achieved by mixing of 5 mg/ml of the CPG2 protein with a 3-fold molar excess of PEGylation reagent Y-shape (Y-SCM-40K), with rapid and thorough shaking by vortex followed by incubation at 30 °C in an incubator shaker at 200 rpm for 10 hours. The PEGylated CPG2 mixture was then subjected to Ni-NTA purification to remove the excess non-reactive PEGylation reagent and further purified by gel filtration using an AKTA purifier. Finally, the purified PEGylated CPG2 conjugate was isolated and quantified.

Determination of PEGylated and HSA conjugated CPG2 catalytic enzyme kinetics

The glucarpidase activities of the pure recombinant HSA-CPG2 (≈2 μg/ml) and PEG-CPG2 (≈2 μg/ml) was determined by measuring methotrexate hydrolytic activity using Tris buffer (0.1 M Tris-HCl pH 7.3 and 0.2 mM ZnSO4) containing Methotrexate (MTX) (0.27 mM) as substrate and measured spectrophotometrically using Plate Reader Infinite M200 PRO NanoQuant (TECAN) at 320 nm, 37 °C for 1 hour.

Kinetic studies of these modified CPG2 (HSA Xen-CPG2 and PEG Xen-CPG2 of ≈2 μg/ml), were also assayed by testing their activities at different MTX concentrations (30-420 μM) in the required Tris-$ZnSO_4$ buffer using Nunc 96 plates with UV transparent flat bottom wells, to determine the stability constant (Km) and the rate of reaction (Vmax). All reactions were carried out at 37 °C for 2 min and the decrease in absorbance at 320 nm was monitored using Plate reader, Infinite M200 PRO NanoQuant. The Michaelis-Menten equation was used for determination of the actual values of Km, Kcat, and Vmax of each protein using GraphPad PRISM 6 software.

One unit of the enzyme represents the amount of enzyme in mg required for hydrolysis of 1 mM of MTX per min at 37 °C. The enzyme activity per ml of protein was calculated using 8,300 as the molar extinction coefficient for MTX.

Circular Dichroism and secondary structure analyses of the modified CPG2

a. Pre-CD Scanning

Purified preparations of the CPG2 (HSA Xen-CPG2 and PEG Xen-CPG2) proteins were dialyzed against Milli-Q water 4 times each for 18 hours, followed by centrifugation for 30 min. at 4 °C. A NanoDrop 2000 spectrophotometer (Thermo Scientific) was used for measuring the protein concentration and the required concentration for CD measurement for each protein was adjusted to about 8-10 µM. The extinction coefficients were taken as ε = 66305 and 23380 M^{-1} cm^{-1} for HSA Xen-CPG2 and PEG Xen-CPG2, respectively.

b. Circular dichroism (CD)

Measurements were made using Chirascan™ Plus CD Spectrometer (Applied Photophysics). CD scanning of the modified CPG2 in far UV spectral region was measured using a SUPRASIL Quartz cuvette demountable rectangular (Hellma®) of 0.2 mm light-path length (sample volume ~70 µl). Scans were made from 260 to 180 nm. All proteins were tested at conc. 8-10 µM at 20 °C. The applied CD parameters were as follows: bandwidth 1 nm and scan time per point of 0.5 sec. Four scans were taken per one sample, and these readings were averaged and smoothed using the CD analysis software. The produced spectra were subtracted from an averaged CD spectra of the used blank (Milli Q water) baseline.

c. CD- deconvolution method

Protein secondary structure of the pure recombinant modified CPG2 were calculated by CD data deconvolution analysis using the CDNN (version 2.1) software tool. The Deconvolution calculation was carried out in the spectral range of (180–260 nm). The parameters used in the deconvolution calculations, the number of residues and molecular weight were taken as 1018 AAs, with 112.38344 kDa for HSA Xen-CPG2 and 81.76148 kDa and 392 AAs for PEG Xen-CPG2 respectively, and 0.02 cm light-pathlength of the cuvette was used.

Mass Spectra analysis of HSA-glucarpidase fusion protein

The purified fusion protein was analyzed on 8% SDS-PAGE electrophoresis. The extracted band-containing HSA-glucarpidase protein was subjected to protein digestion with Trypsin Gold (mass spec grade, Promega) using in gel digestion protocol according to manufacturer's instruction.

The digested proteins were then analyzed by tandem mass spectrometry (LC-MS/MS) using LC/MS LTQ-Orbitrap Elite.

Extracted data was analyzed to identify the protein sequence against protein database using PEAKS Studio software.

CPG2 and its modified forms stability assay

The structural stability of resulting purified proteins was determined by incubation of the 0.1µg/µl purified free Xen-CPG2, PEG Xen-CPG2 and HSA Xen-CPG2 with human serum samples from a normal donor at 37 °C. Samples collected every 5 days for 15 days and analyzed by western blotting. Samples (1µg/lane) were separated on non-denatured gels (native-PAGE) and transferred to nitrocellulose membranes electrostatically. The resulting membranes were blocked with 5% non-fat milk in 1x PBS for 1 hour at room temperature, then incubated with rabbit anti Xen-CPG2 antibody (1:2000) in 1% non-fat milk for 1 hour at room temperature. Following washing, the membranes were incubated with a secondary antibody (horseradish peroxidase conjugated mouse anti-rabbit antibody), the antibody binding detected with ECL chemiluminescent detection reagent as described by the supplier (GE Healthcare).

A further serum stability assay was carried out to investigate the functional stability of the resulting CPG2 proteins. 0.1µg/µl of purified free CPG2, PEG Xen-CPG2and HSA Xen-CPG2 proteins were incubated with serum samples from a normal donor at 37°C for more than12 days. A sample was taken every 2 days and the catalytic activity of the glucarpidase moieties was measured using the MTX hydrolysis assay described above. The percentage of remaining activity at each point was calculated and plotted against the time of sample taking.

Ex-vivo immunogenicity

The immunogenicity of the resulting purified proteins was investigated using a proliferation assay for human peripheral blood mononuclear cells (PBMCs) from normal healthy donors. Total blood samples were collected and the PBMCs separated using ficoll solution (Sigma) and density centrifugation. The isolated PBMCs were cultured at 1 x 10^6 cells/ml of X-vivo medium in 96 well plates. Cells were incubated for 48 hours at 37 °C with the purified endotoxin-free proteins (free Xen-CPG2, PEG Xen-CPG2, and HSA Xen-CPG2) (10µg/ml). Pierce LAL Chromogenic Endotoxin Quantitation Kit was used to detect the level of endotoxin in the purified CPG2 protein samples and, where necessary, endotoxin levels were reduced to > 0.1EU/ml using Pierce™ High Capacity Endotoxin Removal Resin. Following incubation for 48 hours, 10µl of Cell Counting Kit-8 solution (CCK-8) (Sigma) was added to each well and incubated for 3 hours at 37 °C. The absorbance of resulting color was measured at 450 nm using Infinite M200 PRO NanoQuant Plate Reader. Endotoxin level of the resulting purified proteins was lowered to <0.1EU/ml using Pierce™ High Capacity Endotoxin Removal Resin, followed my measuring endotoxin level using Pierce™ LAL Chromogenic Endotoxin Quantitation Kit.

Statistical analysis

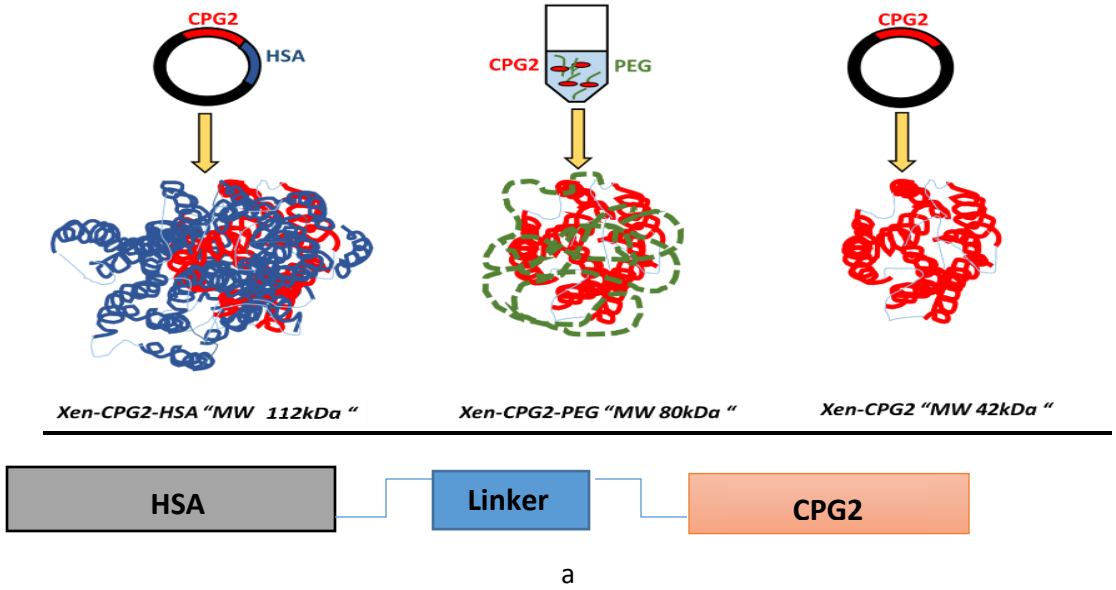
The resulting data are presented as means a ± standard deviation of the means from at least three independent experiments. Significance was obtained by statistical analysis using student t-test (two-tailed). Graph Pad Prism software was used for all analysis and the significance level was set at ≤0.05.

Results

Production of CPG2 Modifications

a. HSA fusion protein design, expression, and purification

The Xen-CPG2 gene was fused in-frame to the 3' end of a gene encoding HSA (Fig 2a) as described in the Experimental Section. The fusion gene was then sub-cloned into the pET28a expression vector and transformed into E. coli BL21 (DE3)RIL. Following induction of expression, recombinant HSA Xen-CPG2 protein was purified using Ni-affinity chromatography. SDS-PAGE suggested that the protein was >90 % pure (Fig 2b).



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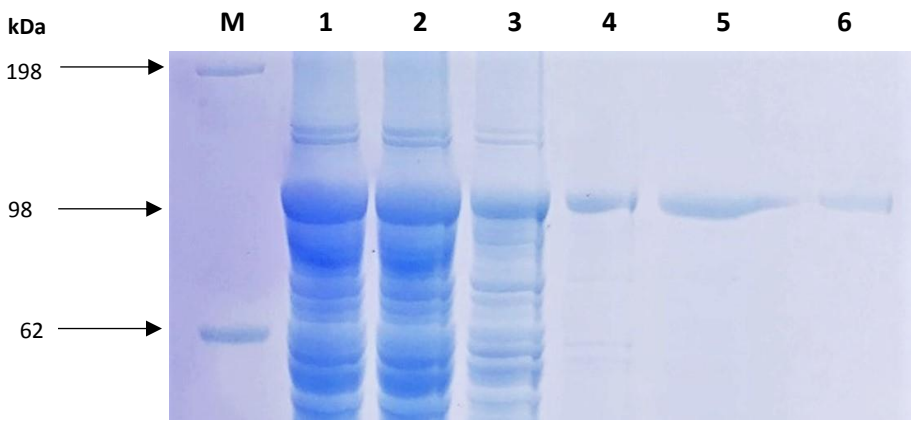
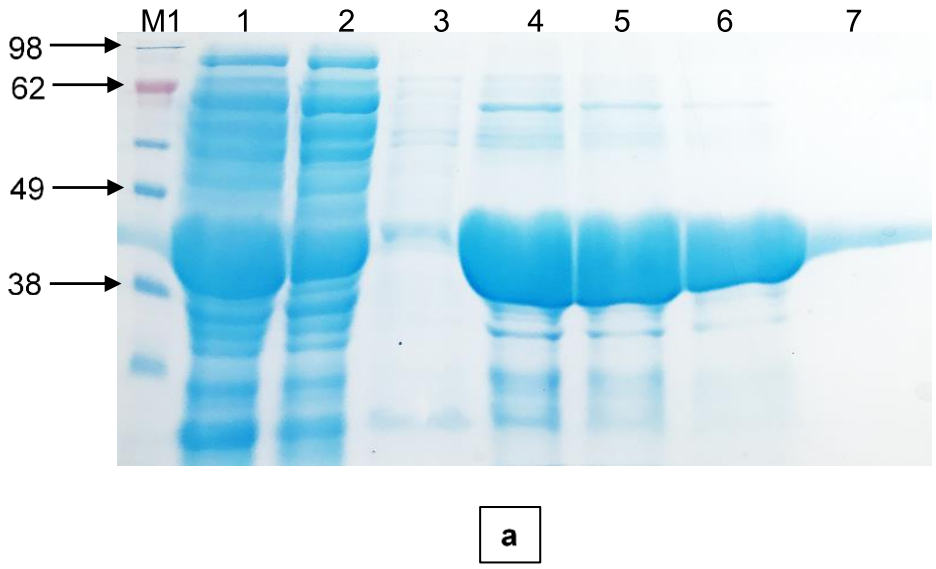


b

Fig 2. Design and purification of the HSA-Xen-CPG2 fusion protein. **a)** Standard recombinant DNA techniques were used to create an in-frame fusion between the 5' end of the Xen-CPG2 gene and the 3' end of the human HSA gene. A sequence encoding a linker (YGGGGSGGGGSGGGG) was inserted between the HSA and CPG2 genes. **b)** The HSA Xen-CPG2 fusion protein was expressed in *E. coli*, purified using Ni-NTA affinity chromatography, and analysed by SDS-PAGE. Lane M showed the SeeBlue Plus2 Pre-stained Protein Standard (198-3 kDa). Lane 1, total soluble protein; lane 2, flowthrough during Ni-NTA affinity purification; lane 3, proteins released during washing of Ni-NTA beads; lanes 4-6, eluted fractions. Proteins were visualized by Coomassie blue staining, indicating >90% purity of the eluted protein. As expected, the fusion protein had an MW of ≈ 112 kDa.

b. PEGylation technology of Xen-CPG2

To extend the half-life and protein stability of Xen-CPG2, and hence improve ADEPT and cancer treatment, polyethylene glycol (PEG) chains were tethered to Xen-CPG2 using PEGylation technology, thereby increasing its hydrodynamic size. PEGylation was achieved by employing a Y-shaped (MPEG20K)2 succinimidyl carboxymethyl ester of MW 40,000 (JenKem Technology) that reacts with the amino groups of lysine side chains on the target protein. To reduce the formation of different PEGylated chain lengths on the protein and to maximize the degree of protein PEGylation, the reaction condition was first optimized in trials that systematically varied the time of exposure to the PEGylation reagent. The PEGylated protein was then purified by Ni-affinity chromatography and further purified by gel filtration prior to comparison with non-PEGylated CPG2 by SDS-PAGE analysis (Fig 3, Appendix A).



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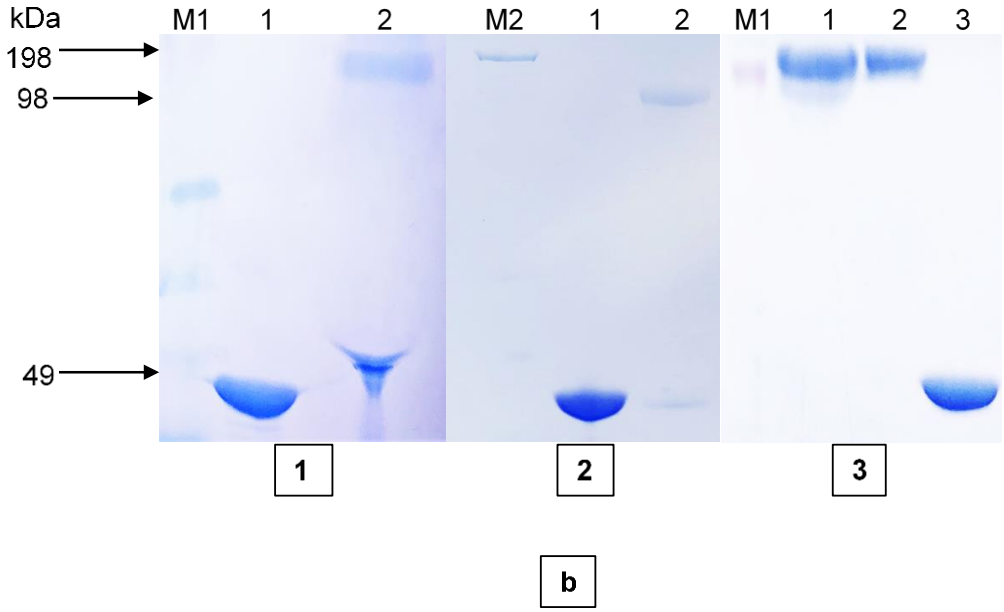

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328 **Fig 3.** PEGylation of Xen-CPG2 using SCM reagent.

329 **a)** SDS-PAGE of Xen-CPG2 at different stages of purification. Xen-CPG 2 was purified as described in the
330 Materials & Methods section. Lane M1 shows a SeeBlue Plus prestained protein marker (3 to 198 kDa) while lane
331 M2 showed a PageRuler Unstained Protein Ladder (10 kDa to 200 kDa). Lane 1, total soluble protein; lane 2,
332 flowthrough during Ni-NTA affinity purification; lane 3, proteins released during washing of Ni-NTA beads; lanes
333 4-7, eluted fractions.

334 **b)** SDS-PAGE of Xen-CPG2 at different stages of PEGylation. Panel 1. Lanes 1 and 2, pure non-PEGylated Xen-
335 CPG2 and PEGylated Xen-CPG2, before removal of PEGylation reagent. Panel 2. Lanes 1, and 2, pure non-
336 PEGylated Xen-CPG2 and PEGylated Xen-CPG2, respectively, after removal of PEGylation reagent by affinity
337 purification. Panel 3. PEGylated Xen-CPG2 and non-PEGylated CPG2 after purification and concentration. Lanes 1
338 and 2, PEGylated Xen-CPG2 at two different concentrations; lane 3,non-PEGylated Xen-CPG2.

The purity of the resulting purified protein is crucial for further investigations as the presence of any non-modified, native CPG2 might interfere and affect the end result. Thus, the purity of the PEGylated- and HSA-fused CPG2 was confirmed by western blot analysis (Fig 4). The results indicated that the engineered and purified proteins were suitable for application studies.

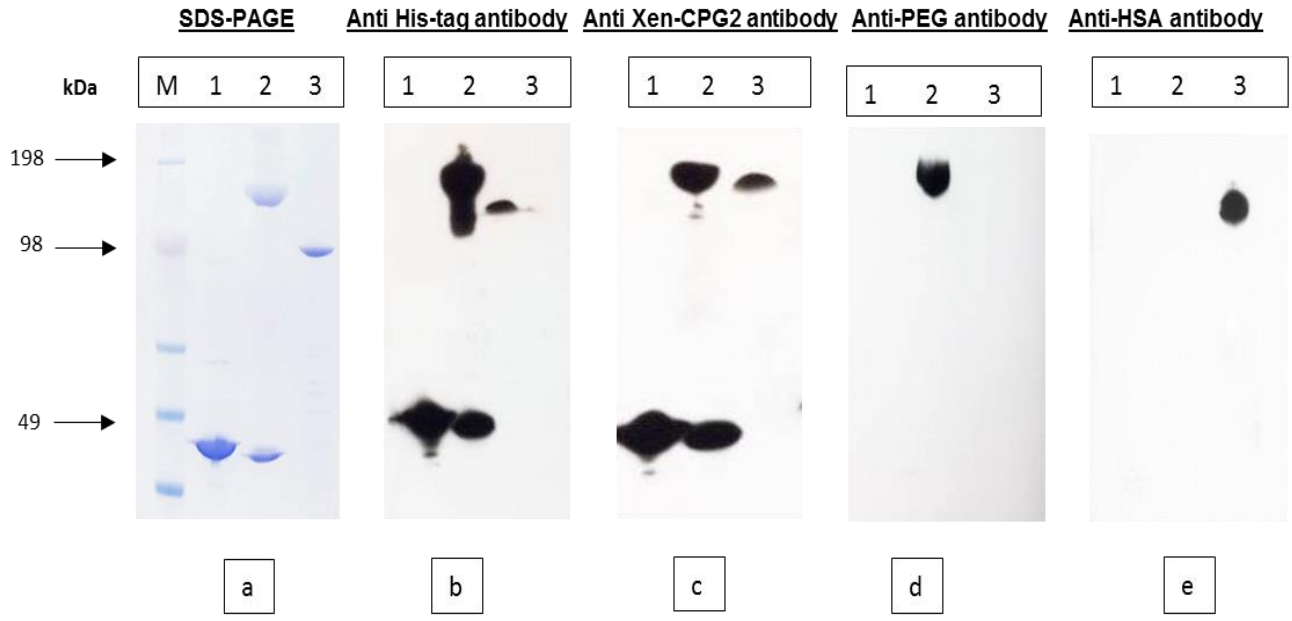


Fig 4. Western blot analyses of different forms of CPG2 relative to the wt. Lane M, SeeBlue Plus2 Prestained ladder (198-10 kDa), while lanes 1-3 are free Xen-CPG2, PEGylated Xen-CPG2 and HSA-Xen-CPG2, respectively. Panel a shows SDS-PAGE of the three purified proteins while panels b-e show the results of immunoblots with anti-His-tag antibody, anti-CPG2 antibody, anti-PEG antibody and anti-HSA antibody, respectively.

PEGylation and HSA-fusion affects the hydrolytic activity of CPG2

E. coli cells expressing the HSA-CPG2 fusion protein were tested for glucarpidase activity by assessing the degree of folate hydrolysis using agar plates supplemented with folate in the growth medium. Colonies were dark orange color and were surrounded by clear zones suggesting that fusion with HSA did not grossly affect the glucarpidase activity of CPG2 (Fig 5).

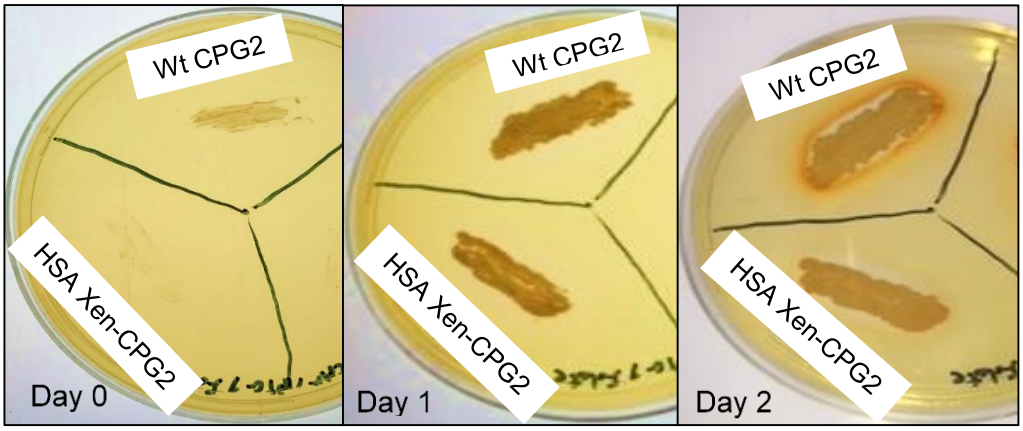


Fig 5. Development of folate-specific hydrolytic activity of *E. coli* expressing recombinant fused HSA-Xen-CPG2 or wild-type Xen-CPG2. Cells were inoculated on LB agar supplemented with folate and IPTG, and the relevant antibiotics. Their growth and coloration were recorded at daily intervals.

The catalytic activity of PEGylated- and HSA-fused CPG2 proteins was further investigated by measuring the rate of MTX hydrolysis by the purified proteins (Fig 6). The derived kinetic parameters for Xen-CPG2, PEG-Xen-CPG2 and HSA-Xen-CPG2 were, respectively: V_{max} values: 24.35 ±1.91, 20.69 ±1.428, 48.72 ± 4.389 µM/min; K_m values, 50.56 ± 10.71, 69.97 ± 17.12, 66.14 ± 21.85 µM; and K_{cat} values: 11.49 ± 0.1947, 9.759 ± 0.2093, and 8.4 ± 0.2401 S^{-1}.

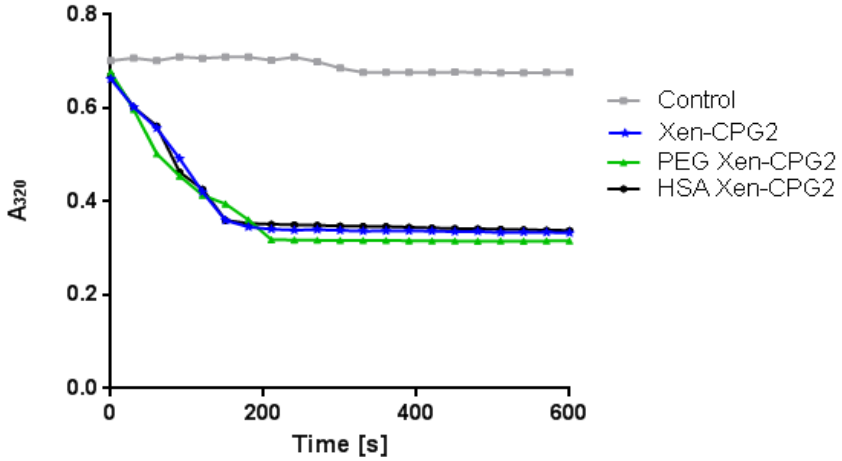


Fig 6. Activity assay of different forms of Xen-CPG2 on hydrolysis of methotrexate (MTX). The activity of free Xen-CPG2 (blue), the activity of HSA-Xen-CPG2 (black) and activity of PEG-Xen-CPG2 (green) relative to the buffer control (line in gray). See Materials & Methods for further details.

PEGylation and HSA conjugation enhance the structural and functional stability of CPG2

To investigate the stability of the PEGylated and HSA-conjugated CPG2 proteins, they were incubated with serum from healthy donors at 37^{o}C. Samples were taken every 2 days and tested for CPG2 enzymatic activity. The resulting remaining percentage activity (Fig 7) indicates that PEGylated and HSA conjugated CPG2 proteins retained more than 50% of their enzyme activity after 14 days. In contrast, Xen-CPG2 retained less than 40% of its activity.

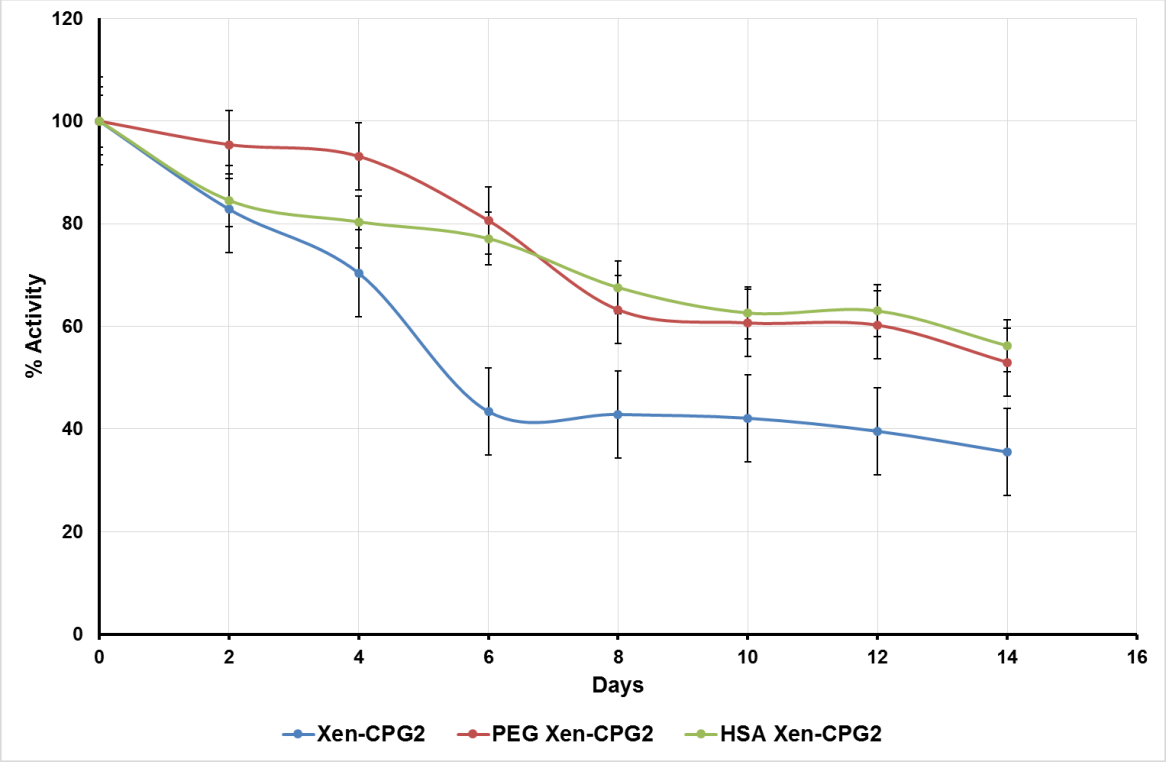


Fig 7. Remaining catalytic activity of different CPG2 variants following incubation in human serum. The proteins (0.1 mg/ml) were incubated in serum at 37oC for 14 days and the remaining methotrexate hydrolysis activity was measured at 48 hour intervals.

The results suggest that genetic fusion of CPG2 with HSA was better at stabilizing CPG2 activity in serum (with ~60% of activity maintained at day 14), compared with the PEGylated form. To further investigate the structural stability of the proteins, samples were incubated in serum prior to separation by native PAGE and analysis by immunoblotting using anti-CPG2 antibodies. Free CPG2 was progressively degraded as the incubation time increased from Day 10 to Day 15 (Fig 8). In contrast, PEGylated and HSA-conjugated CPG2 remained relatively intact from Day 0 to Day15, indicating that the engineered proteins have enhanced stability in serum (Fig 8).

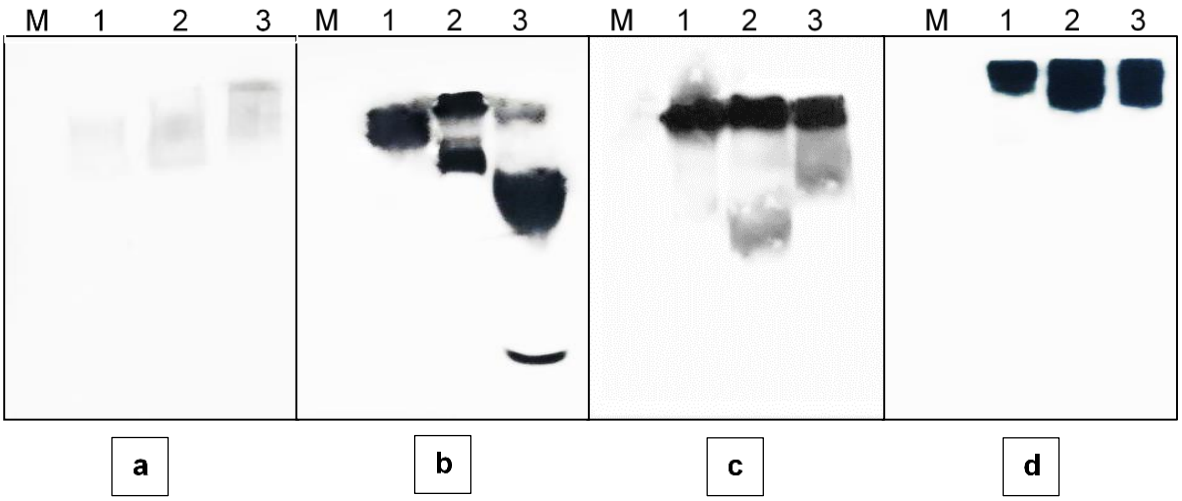


Fig 8. Stability of different CPG2 variants in normal human serum. The proteins were incubated in serum and samples were taken on days 0, 10 and 15. The samples were separated by native PAGE and analyzed by western blotting using anti Xen-CPG2 antibody. Lane M, SeeBlue Plus2 Prestained ladder (198-10 kDa); lanes 1, 2, and 3 , samples taken on days 0, 10, and 15, respectively. Panel a: serum only as control; panel b: Xen-CPG2; panel c: PEG-Xen-CPG2; and panel d: HSA-Xen-CPG2.

Circular dichroism spectral analysis

Circular dichroism (CD) spectroscopy was used to obtain information on how PEGylation or fusion with HSA might affect the overall structure of CPG2 (Fig 9). CD is based on the principle that the differential absorption of polarized light by a chiral molecule (i.e. right- and left-handed rotation of circularly polarized light induced by optically active molecules in the sample) provides structural information. The obtained CD data was deconvoluted to give the predicted secondary structure as shown in Table 1.

Table 1 - Comparison of secondary structures of Xen-CPG2, PEG-CPG2, and HSA-CPG2

Protein / Secondary structure component (%) [1]	Xen-CPG2	PEG-CPG2	HSA-CPG2
Alpha helix	69.3	81.7	30.9
Anti-parallel	1.2	0.6	11.1
Parallel	3.2	1.6	8.9
Beta-turn	11.3	9.1	17.4
Random coil	14.9	6.7	31.5
Total Sum	99.9	99.7	99.8

[1] The relative amounts of different secondary structures in Xen-CPG2, PEG-CPG2, and HSA-CPG2 were calculated from the spectral data shown in Fig 8 by CDNN deconvolution analysis. The average values of each secondary structure component following four sets of measurements is shown as percentages.

The percent composition of the four main secondary structure components calculated by CDNN deconvolution analysis of the obtained spectra (Fig 9.), consists of 69.3% alpha helix, 1.2% antiparallel, 3.2% parallel, 11.3% beta turn, and 14.9% random coil for Xen-CPG2 [24] . In contrast, and 81.7% helix, 0.6% antiparallel, 1.6% parallel, 9.1% beta turn, and 6.7% random coil for PEG-CPG2 and 30.9% parallel, 11.1% antiparallel, 8.9% parallel, 17.4% beta turn and 31.5% random coil for HSA-CPG2, respectively.

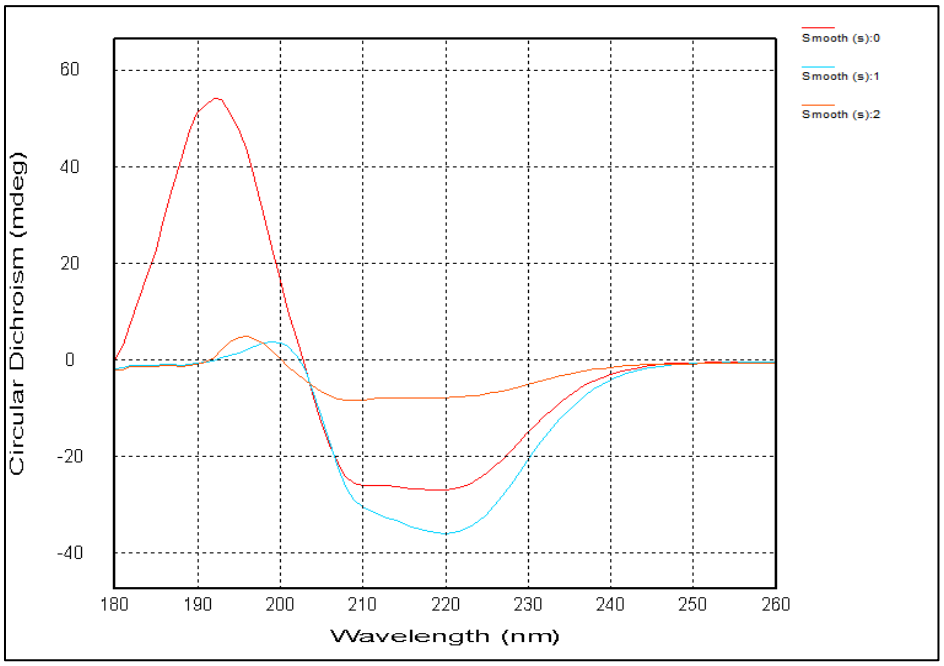


Fig 9. Far UV spectroscopy of free Xen-CPG2, PEG-Xen-CPG2, and HSA-Xen-CPG2 using CHIRASCAN. The lines labeled Smooth 0, 1, and 2 represent the CD spectra for Xen-CPG2, PEG Xen-CPG2, and HSA Xen-CPG2, respectively

In vitro immunogenicity of PEGylated and HSA conjugated CPG2 proteins

The immunogenicity of the produced proteins was examined using PBMCs from healthy donors. The proliferation of the cells was detected following incubation with either the vehicle (negative control), LPS (positive control) or the endotoxin low produced proteins (CPG2, PEGylated, and HSA conjugated Xen-CPG2). As shown in Fig 10. PEG-Xen-CPG2 was significantly low in immunogenicity compared to the positive control. Fusion with HSA also lowered CPG2 immunogenicity significantly in most of the samples.

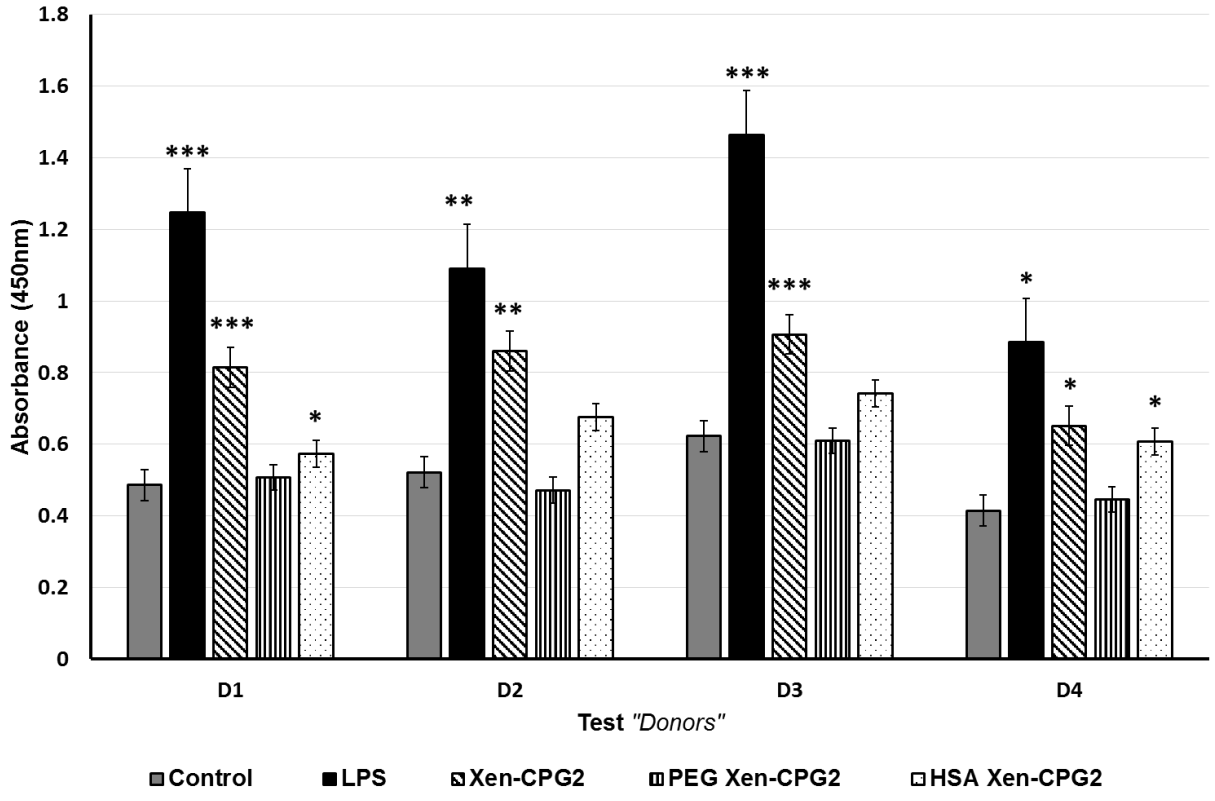


Fig 10. Human PBMC proliferation assay (Immunogenicity). PBMC from healthy donors incubated with purified proteins followed by determining their proliferation. A significant increase was found in the positive control, Lipopolysaccharide (LPS) compared with all control groups with the vehicle only. When comparing with negative control, PEG Xen-CPG2 treated cells in all groups showed no significant increase in proliferation, whereas HSA

Xen-CPG2 treated cells showed similar result except in two donors. ** $P < 0.01$, *** $P < 0.0001$, an unpaired test was used to analyze data. $P < 0.05$ was considered statistically significant.

Discussion

Antibody directed enzyme prodrug therapy (ADEPT) is an effective strategy for targeted cancer treatment. The technique, however, suffers from two significant drawbacks; namely, the patient immune system will progressively generate antibodies against the enzyme due to the repeated injection of CPG2, thereby limiting its efficacy, and the enzyme will be liable to degradation by the patient's proteases.

In our previous work [13] we isolated a novel glucarpidase whose raised antibodies did not cross-react with the one in clinical use. In principle, therefore, it would be possible to delay the production of antibodies in a patient by alternating the use of the two versions of CPG2. However, this strategy may not totally solve the problem and in any case does not address the issue of limited protein stability in a patient's blood, e.g. due to proteolytic degradation [26]. In this study, therefore, we adopted a different approach to produce biobetter glucarpidases, which should both reduce the potential for antibodies production and also protect the protein against endogenous proteases.

PEGylation of proteins and other biomolecules is one of the most effective strategies to produce biobetter therapeutics. It improves the pharmacokinetics and pharmacodynamics of the conjugated molecules in relation to the non-conjugated one, increases water solubility and also protects against proteolytic degradation. On the other hand, human serum albumin has a long half-life in the body and proteins fused to this protein also have extended half-lives [27, 28].

Modifications of glucarpidase by PEGylation or by genetic fusion to HSA is thus a promising strategy to allow these molecules to be used optimally in drug detoxification or in ADEPT.

The data presented in Figs 2-6 and appendix A indicate that the production of pure and active PEG-CPG2 and HSA-CPG2 was successfully achieved. The mass spectra analysis confirmed the formation of the HSA-CPG2, Appendix B. However, it was important to verify that the conjugated forms of CPG2 retained enzyme activity – it remained possible that attachment of large additional molecules might sterically hinder access to the active site of CPG2. Surprisingly, enzyme activity studies indicate that HSA-CPG2 has slightly increased catalytic activity relative to free CPG2 – the V_{max} of the former was 48.72 ±4.389 µM/min whereas unconjugated CPG2 has a V_{max} of 24.35 ±1.91 µM/min [13]. In the case of PEGylated CPG2, the catalytic activity found to be slightly lower than that of unconjugated CPG2 (Vmax value of PEG-CPG2: 20.69 ±1.428 µM/min). Thus, the attached PEG may slightly restrict the access of the substrate to the enzyme active site or slightly alter the enzyme's conformation. The results of far UV spectroscopy (CD) suggest that significant structural changes in secondary structure components of each

protein from induced by PEGylation and/or HSA conjugation compared with the CD scan and its deduced secondary structure results of free Xen-CPG2 recently published by our group [13].

To compare the stability of the modified forms of CPG2 with the free enzyme, we incubated them with human blood serum for a total of 14 days. Fig 7 and fig 8 confirm that both HSA-CPG2 and PEG-CPG2 are significantly more stable than free CPG2 and that they also have longer half-lives. In the latter case, PEGylation (incorporation of PEG negative charged molecules) is known to stabilize the conformation of proteins by increasing the intramolecular hydrogen bonding and increasing the hydrophilicity [29, 30]. Increasing protein conformation also restricts access of proteases to the conjugated protein. On the other hand, HSA, which is well known to be non-immunogenic and biocompatible, also extends the half-life of the HSA-protein therapeutics [31].

A PBMC proliferation assay, where T-cell (in the PBMCs) proliferation and differentiation is induced upon exposure to their cognate antigens, is considered to be a helpful tool during preclinical safety assessment and is used efficiently to evaluate and predict immunological effects of biopharmaceuticals [32]. To assess the potential immunotoxicity of the modified CPG2s relative to the free CPG2, we, therefore, carried out an *ex-vivo* lymphocyte proliferation assay. Our results (Fig.9) indicated that in case of PEG-CPG2 no significant induction of T-cell proliferation was observed, indicating that the attached PEG molecules are able to mask the immunogenic epitopes in CPG2 that react with immune cells and induce immunogenicity. In contrast, HSA-CPG2 triggered a significant increase in T-cell proliferation compared with the negative control. This unexpected result, which apparently contradicts the protein stability results (Figs 7 and 8), could be explained by the presence of endotoxin impurities, which might induce immunogenicity. We, however, carried out further purification steps to remove any remaining endotoxin but obtained the same results. Further study is needed to establish the cause of the observed immunogenicity.

Collectively, our results show that conjugation of CPG2 with HSA or with PEG has a significant effect on CPG2 structural and functional stability *in vitro* and *ex-vivo*. Furthermore, PEGylated CPG2 was shown to have a reduced immunogenic effect on PBMCs compared with free CPG2. Our findings pave the way for *in vivo* studies and possible clinical investigation using modified forms of CPG2.

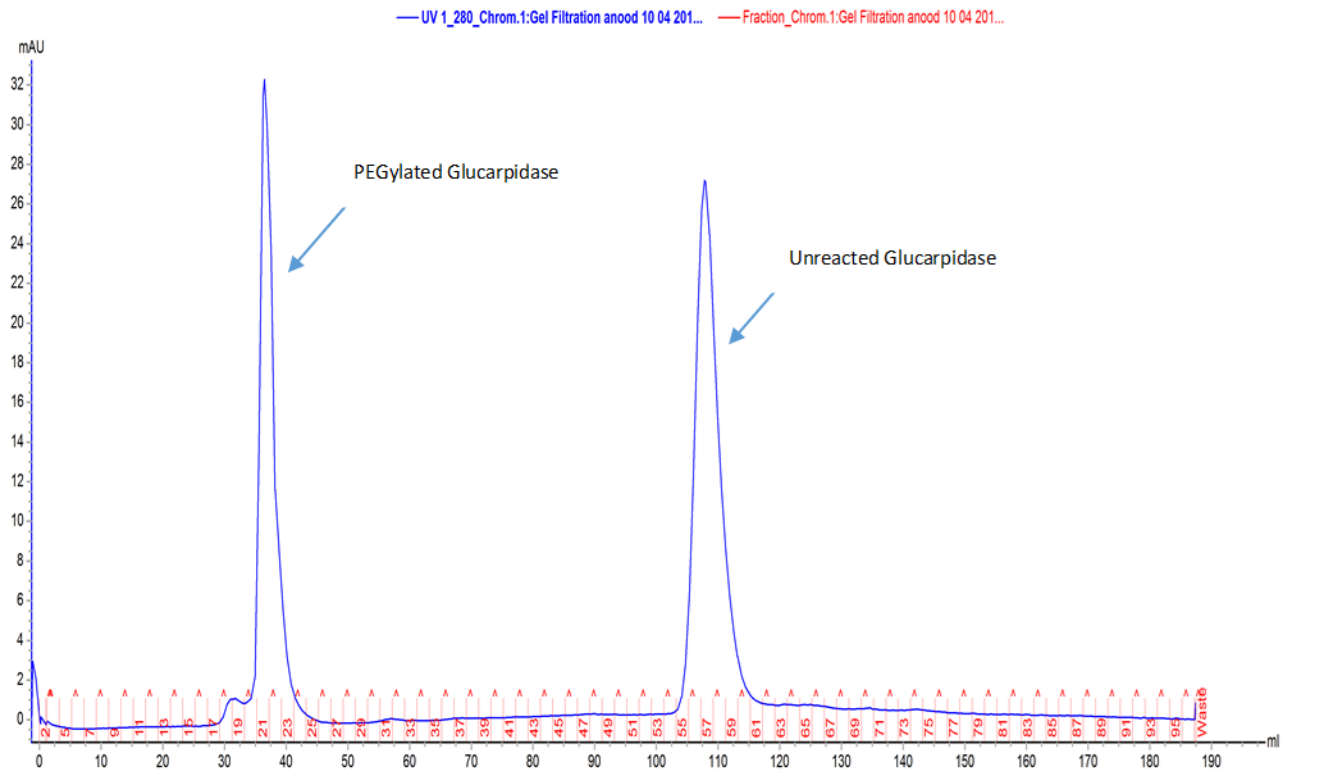
Conclusion

Glucarpidase (CPG2) is an important enzyme used in antibody directed enzyme prodrug therapy (ADEPT) for cancer treatment but with several pitfalls such as host immune response and proteases degradation. The Human serum albumin and PEG molecules are good half-life extenders with several

beneficial features, such as resistance to proteases degradation and low immune response. In this work we successfully carried out conjugation of HSA and PEG molecules to our newly isolated glucarpidase (CPG2), generating two novel glucarpidase conjugates, HSA-CPG2 and PEG-CPG2. We demonstrated that both HSA and PEG molecules could be conjugated to glucarpidase without compromising the critical property of the target protein such as enzyme activity. Our work shows that both glucarpidase conjugates have serum half-lives much higher than the free glucarpidase. We also show that PEG-CPG2 have a reduced immunogenic effect on PBMCs compared with free CPG2. Our work paves the way for the *in vivo* clinical investigation and clinical trials using our novel modified forms of CPG2 for cancer treatment and drug detoxification.

Appendices

Appendix A



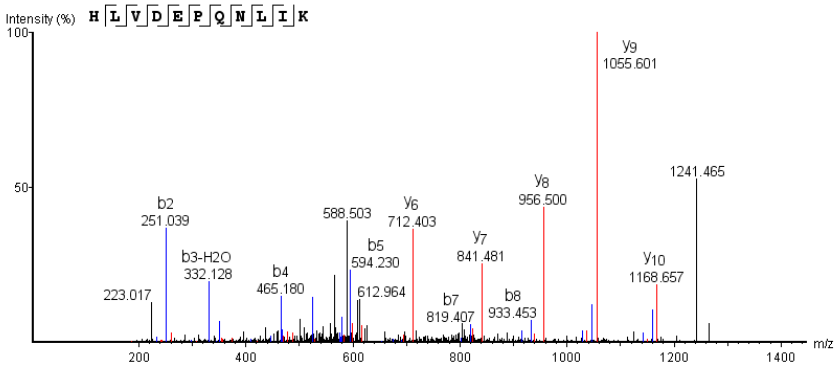
Appendix A.1: Size exclusion of PEG-CPG2 under high-resolution conditions using AKTA purifier.

Appendix B

Protein List

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	#Peptides	#Unique	PTM	Avg. Mass	Description
4	1	P02768|ALBU_HUMAN	287.76	47	35	7	Y	69367	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
40	20750	P02769|ALBU_BOVIN	194.30	13	10	3	Y	69294	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
109	20920	P06621|CBPG_PSES6	146.32	16	7	7	Y	43932	Carboxypeptidase G2 OS=Pseudomonas sp. (strain RS-16) GN=cpg2 PE=1 SV=1
total 3 proteins									

Best Unique PSM (Scan 5107, m/z=653.3622, z=2, RT=27.24, ppm=0.8):



P06621|CBPG_PSES6

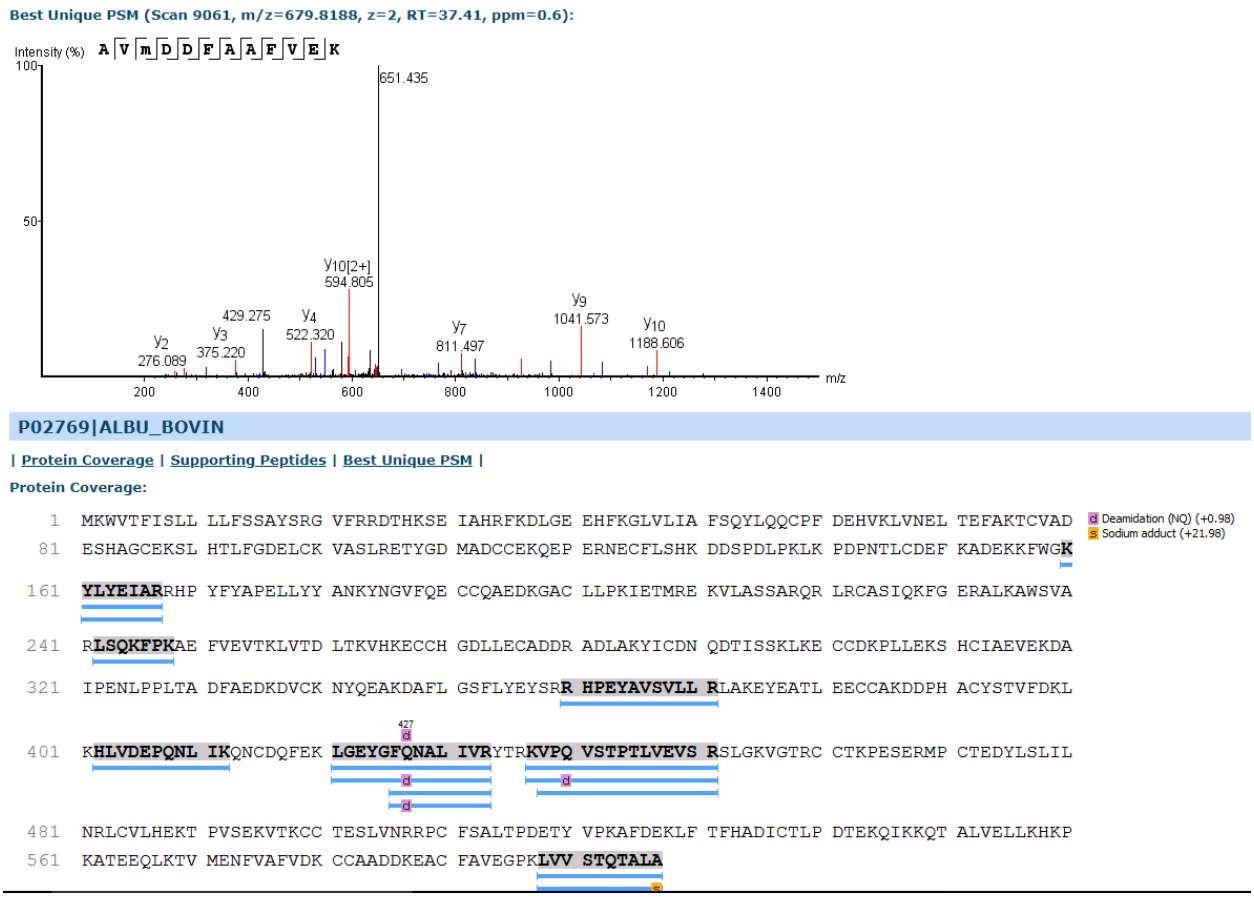
| Protein Coverage | Supporting Peptides | Best Unique PSM |

Protein Coverage:

```
  1  MRPSIHRTAI AAVLATAFVA GTALAQKRDN VLFQAATDEQ PAVIKTLEKL VNIETGTGDA EGIAAAGNFL EAELKNLGFT
 81  VTRSKSAGLV VGDNIVGKIK GRGGKNLLLM SHMDTVYLKG ILAKAPFRVE GDKAYGPGIA DDKGGNAVIL HTLKLLKEYG
161  VRDYGTITVL FNTDEEKGSF GSRDLIQEEA KLADYVLSFE PTSAGDEKLS LGTSGIAYVQ VNITGKASHA GAAPELGVNA
241  LVEASDLVLR TMNIDDKAKN LRFNWTIAKA GNVSNIIPAS ATLNADVRYA RNEDFDAAMK TLEERAQQKK LPEADVKVIV
321  TRGRPAFNAG EGGKKLVDKA VAYYKEAGGT LGVEERTGGG TDAAYAALSG KPVIESLGLP GFGYHSDKAE YVDISAIPRR
401  LYMAARLIMD LGAGK
```

Formylation (+27.99)
Oxidation (M) (+15.99)
Mutation

Appendix B.1: Mass spectra and matching peptide sequences for Carboxypeptidase G2 (matching peptides are highlighted)



Appendix B.2: Mass spectra and matching peptide sequences for human serum albumin (matching peptides are highlighted)

Acknowledgment

- QNRF grant number NPRP6-065-3-012, Qatar National Research Fund, Doha Qatar for funding this work with grant number NPRP No.:NPRP6-065-3-012.
- Professor C. David O'Connor, Xi'an Jiaotong-Liverpool University for reading and reviewing the manuscript.
- TMPL Lab, Anti-Doping Lab, Doha, Qatar for carrying out the mass spectra analysis of HSA-glucarpidase

Ethics approval and consent to participate

The study was approved by the Anti-Doping Lab-Qatar Institutional Review Board, Ethical approval number: E2017000205. All blood samples used in this study were taken from authors involved in the work.

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